The ancient drug salicylate directly activates AMP-activated protein kinase

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Supplementary information

Legends to Supplementary Figures

Figure S1: Evidence that the effect of salicylate is not mediated by the Ca^{2+}-CaMKK pathway. (A) Effect of salicylate or A23187 ± STO-609. (B) Effect of salicylate or A23187 ± STO-609 on AMPK phosphorylation. HEK-293 cells were incubated in duplicate dishes with or withour 25 μM STO-609; results in (A) show means of duplicates that agreed to within 10%.

Figure S2: Salicylate has no effect on the activity of PP2Cα measured using a ^{32}P-labelled peptide substrate. Results are means of duplicate assays. Pilot experiments were performed to ensure that the release of ^{32}P at a fixed time was proportional to the amount of PP2Cα added.

Figure S3: Salicylate and A-769662 cause increasing phosphorylation of (A) AMPK and (B) ACC in hepatocytes isolated from WT but not β1-KO mice. Results are mean ± SEM (n = 3) and are expressed as ratios of absorbance (determined by densitometry) of the pAMPK or pACC bands relative to a glyceraldehyde phosphate dehydrogenase (GAPDH) loading control. Significance of differences was determined by 2-way ANOVA with Bonferroni's test of each treatment versus control (*p<0.05; **p<0.01; ***p<0.001).

Figure S4: (A) Treatment of isolated hepatocytes from WT mice with salicylate up to 10 mM; and (B) treatment of WT mice with salicylate (250 mg/kg) or A-769662 (30 mg/kg), does not change adenine nucleotide ratios. (A) Hepatocytes from WT mice were incubated for 1 hr with the concentrations of salicylate shown, extracted with 0.5 M perchloric acid and analyzed by capillary electrophoresis as described previously (*1*). (B) Liver samples were snap frozen and ground to a powder in liquid N_2, and the powder extracted with 0.5 M perchloric acid and analyzed by capillary electrophoresis (*1*). Results are expressed (mean ± SEM, n = 4 to 8 for hepatocytes, n = 6 or 7 for livers) as AMP/ATP or ADP/ATP ratios (the AMP peak was too small to reliably quantify from hepatocytes). Neither salicylate nor A-769662 caused significant changes in these ratios by 1-way ANOVA.

Figure S5: Treatment of mice with salicylate (250 mg/kg) *in vivo* causes activation and phosphorylation of AMPK in soleus muscle and adipose tissue. Kinase activities (top) were determined using immunoprecipitate kinase assays (*2*) and results expressed as mean ± SEM (n = 6 or 7). Statistical significance of differences from controls without salicylate was assessed using two-tailed t tests: ***p<0.001. Duplicate Western blots of equal protein loadings from two animals are shown at the bottom. Note that the α1 and α2 subunit isoforms of AMPK in mice differ in mass (64 and 62 kDa respectively) and can be resolved by SDS-PAGE; soleus muscle expresses both isoforms whereas adipose tissue expresses mainly α1.

Figure S6: Differences in: (A/B) area under curve of respiratory exchange ratio; and (C/D) carbohydrate utilization, calculated from the experiments shown in Figs. 4C-F. Results are mean ± SEM, n = 6 to 8; significance of differences from controls without drug were determined by 1-way ANOVA, with Bonferroni's test comparing drug treatment with control for each genotype (*p<0.05, **p<0.01, ***p<0.001).

Figure S7: Parameters of glucose homeostasis measured in high fat-fed β1-KO mice and controls. WT and β1-KO mice were fed a high-fat diet starting at 7 weeks of age (45% fat by kcal, Research Diets). After 12 weeks, mice were injected daily with salicylate (250 mg/kg) or an equal volume of saline. After 12-14 days of this treatment, (A) fasting serum glucose and (B) insulin were measured, (C) glucose tolerance tests performed, and (D) homogeneous model assessment-insulin resistance (HOMA-IR) calculated as described (*3, 4*). Results are mean ± SEM, n = 5 to 7; significance of differences was determined by 1-way ANOVA, with Bonferroni's test comparing drug treatment with control for each genotype (*p<0.05, **p<0.01, ***p<0.001). After salicylate treatment, whole-body and epididymal fat pad weights were 44.2 ± 1.6/45.8 ± 1.7, and 2.53 ± 0.16/2.78 ± 0.10 (mean ± SEM in gram, WT/β1-KO mice respectively). These differences were not significant by *t* test.

Experimental procedures

Proteins, antibodies and other materials

Rat liver AMPK was purified to the gel filtration step (*5*). Polycistronic plasmids for expression of the human α1β1γ1 and α1β2γ1 complexes were constructed as described (*6*). All DNA amplifications were carried out using KOD Hot Start DNA Polymerase (Novagen). Mutagenesis was performed using the QuickChange method (Agilent) but with KOD Hot Start DNA Polymerase. AMPK-α1 (NCBI NP_006242.5) was amplified from IMAGE EST 4839033, -β1 (NCBI NP_006244.2) from IMAGE EST 2961281, -β2 (NCBI NP_005390.1) from IMAGE EST 6091476, and -γ1 (NCBI AAH00358.1) from IMAGE EST 2964434. AMPK-β1 and -β2 were cloned into the BamH1 and Not1 sites of pcDNA5FRTTO (Life Technologies). The polycistronic plasmids were expressed in *E. coli*, purified using a HisTrap column (GE Healthcare) and eluted using an imidazole gradient. The eluted protein was dialysed into 50 mM Na Hepes pH 8.0, 150 mM NaCl, and incubated with bacterially expressed CaMKKβ in the presence of MgATP for 30 min at 30 °C. CaMKKβ and unreacted MgATP were removed by size exclusion chromatography and an in-line Glutathione-Sepharose column (GE Healthcare). Recombinant PP2Cα was expressed and purified as described previously (*7*). Antibodies against AMPK-α1 and -α2 were as described (*8*). PKA was expressed and purified as described (*9*). Antibody against pACC were as described (*10*), against pAMPK were from Cell Signalling, against GAPDH and AMPK –γ1 were from Abcam, and against the FLAG epitope were from Sigma. The pan-β antibody was a monoclonal antibody developed by immunizing mice with the bacterially expressed human AMPK (α2β2γ1) complex and selecting clones that recognized β1 and β2. Salicylate, aspirin, A23817 and quercetin were from Sigma, Poole, UK. STO-609 was from Tocris Bioscience, Bristol, UK. A-769662 was synthesized as described previously (*11*).

Animal experiments

β1-KO mice were described previously (*12*); all protocols complied with the McMaster University Animal Ethics Research Board. Hepatocytes were prepared from β1-KO or WT mice and incubated in culture as described (*13*). Hepatic fatty acid oxidation was assessed by measuring the oxidation of [^{14}C]palmitate (Perkin Elmer) as previously described (*12*). Activation of AMPK *in vivo* was assessed

after intraperitoneal injection of salicylate (250 mg/kg) or A-769662 (30 mg/kg) in β1-KO or WT mice. These doses are comparable to those used in previous studies that have observed improvements in insulin sensitivity following chronic treatment in obese rodent models (120 mg/kg salicylate by continuous infusion (*14*); 30 mg/kg intraperitoneal A-769662 (*15*). Tissues were harvested (*16*) after 90 min and stored at -80°C until analysis. Analysis of respiratory exchange ratio was performed using the Oxymax Columbus Instruments Comprehensive Lab Animal Monitoring System; mice were acclimatized to the system for 24 hr prior to data collection. β1-KO and WT mice were fasted overnight and allowed access to food at 07:00. After a re-feeding period, mice were injected with salicylate (250 mg/kg), A-769662 (30 mg/kg) or vehicle (saline for salicylate, 5% dimethyl sulfoxide in phosphate-buffered saline for A-769662) at 09:00. After injection, mice were denied access to food, and respiratory measurements were continued until 19:00. The same cohort was used for all treatments, with one week separating salicylate and A-769662 injections. Rates of carbohydrate ($4.58*VCO_2$ - $3.23*VO_2$) and fat ($1.70*VO_2$ - $1.69*VCO_2$) utilization were calculated as previously described (*17*). Analyses of *in vivo* phosphorylation and expression of AMPK and ACC, and serum non-esterified fatty acids (NEFAs), were performed as described (*4*).

Cell culture

HEK-293 cells stably expressing WT or R531G mutant γ2 have been described (*1*). HEK-293 cells expressing FLAG-tagged human β1 (WT or S108A mutant) or WT β2 from a tetracycline-inducible promoter were made using the Flp-In™ system (Invitrogen) according to manufacturers' protocols. HEK-293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose, 10% (v/v) foetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin and 200 μg/ml hygromycin B. Expression of β1/β2 subunit was induced, 24 hr prior to treatment, by addition of 1 μg/ml tetracycline (Invitrogen). After various treatments as indicated in figure legends, cells were lysed using the rapid lysis procedure (*2*) and lysates stored at -80ºC until use.

Kinase and phosphatase assays

AMPK was assayed using the *SAMS* peptide (Figs. 2A-E) or *AMARA* peptide (all other Figures) as previously described (*2, 18*), substrate except that assays were at 2 mM rather than 200 μM ATP. In

pilot experiments performed at the sub-physiological ATP concentration of 200 μM, we found that although salicylate activated AMPK at concentrations of 0.3 - 3 mM, at concentrations above 3 mM it began to inhibit (not shown). The inhibitory effect could be eliminated by carrying out the assays at 2 mM ATP (Fig. 2A), suggesting that it was due to competition between salicylate and ATP at the catalytic site on AMPK. AMPK from cell lysates (60 μg protein) was assayed in immunoprecipitates made using anti-α1/α2 antibodies or EZview Red anti-FLAG M2 affinity gel (Sigma) as specified. PP2Cα was assayed using a generic kinase peptide substrate (*19*) after phosphorylation using PKA. Briefly, 12 μg of GST-[GGGGRRATV]$_3$, bound to glutathione-Sepharose, was incubated with PKA (2 units) for 30 min at 30ºC in the presence of 5 mM $MgCl_2$ and [γ-^{32}P]ATP (200 μM; 2500 cpm/pmole). After extensive washing to remove the PKA and unreacted ATP the incorporation was determined to be 2.8 moles phosphate per mole substrate. Phosphorylated GST-[GGGGRRATV]$_3$ (50 ng) was incubated with 200 ng of PP2Cα and 5 mM $MgCl_2$ for 10 min at 30ºC in the presence or absence of salicylate at the concentrations indicated (Fig. S1) and the release of ^{32}P measured.

Analysis of dephosphorylation of AMPK in cell-free assays

Bacterially expressed human AMPK (either α1β1γ1, α1β1[S108A]γ1 or α1β2γ1) was incubated with protein PP2Cα (200 ng) ± 5 mM $MgCl_2$, along with either AMP, A-769662 or salicylate as indicated. After 10 min pT172 phosphorylation was determined by Western blotting, and samples were also diluted 75-fold into AMPK assays to determine kinase activity.

SDS-PAGE and Western blotting

SDS/PAGE was performed using precast Bis-Tris 4%–12% gradient polyacrylamide gels in the MOPS buffer system (Invitrogen). Proteins were transferred to nitrocellulose membranes (Bio-Rad) using the Xcell II Blot Module (Invitrogen). Membranes were blocked for 1 hr in Odyssey blocking buffer (Li-Cor Biosciences, Cambridge, UK). The membranes were probed with appropriate antibody (0.1–1 μg/ml) in Odyssey blocking buffer. Detection was performed using secondary antibody (0.2 μg/ml) coupled to IR 680 or IR 800 dye and the membranes scanned using the Li-Cor Odyssey IR imager. Protein concentrations were determined by Coomassie Blue binding (*20*) with bovine serum albumin as standard.

Other analytical procedures

ADP/ATP ratios and cellular O_2 uptake were measured as described previously (*1*).

Presentation of data and tests for statistical significance

Where error bars are not visible in X-Y plots, they are smaller than the symbol used to represent the data point. Assays of effects in cultured HEK-293 cells were of replicate dishes of cells, with each value being the mean of duplicate immunoprecipitate kinase assays. Statistical significances of differences were assessed using GraphPad Prism 5 for Mac OSX, with the specific tests used being given in Figure legends.

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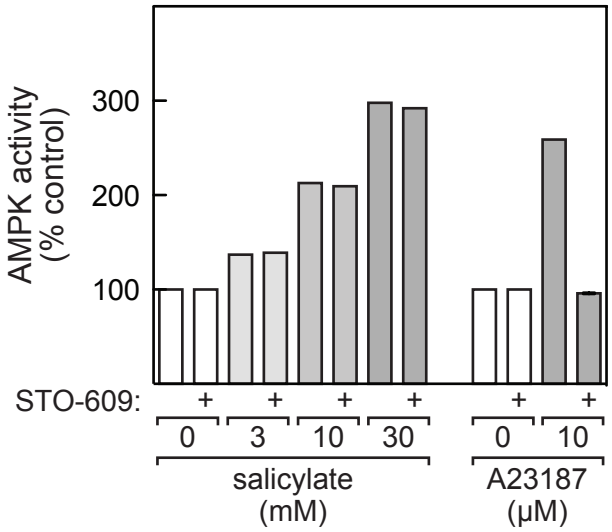
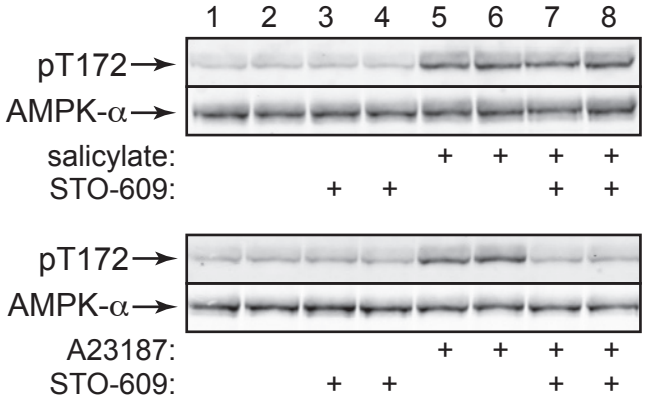
Hawley et al Figure S1

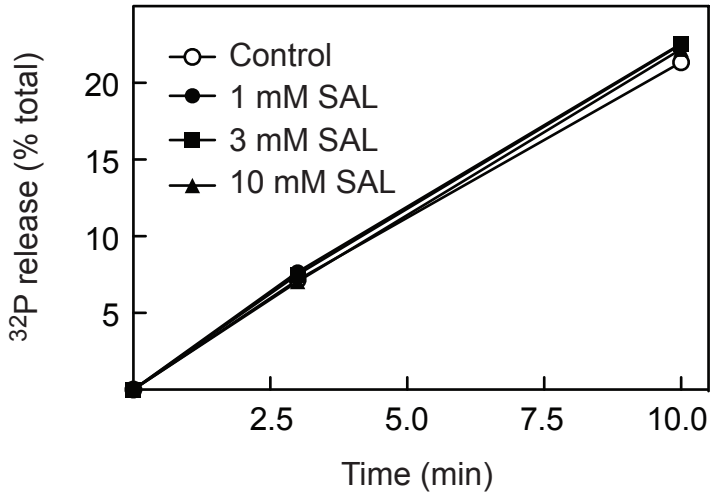
A) Effect of STO-609 on AMPK activation



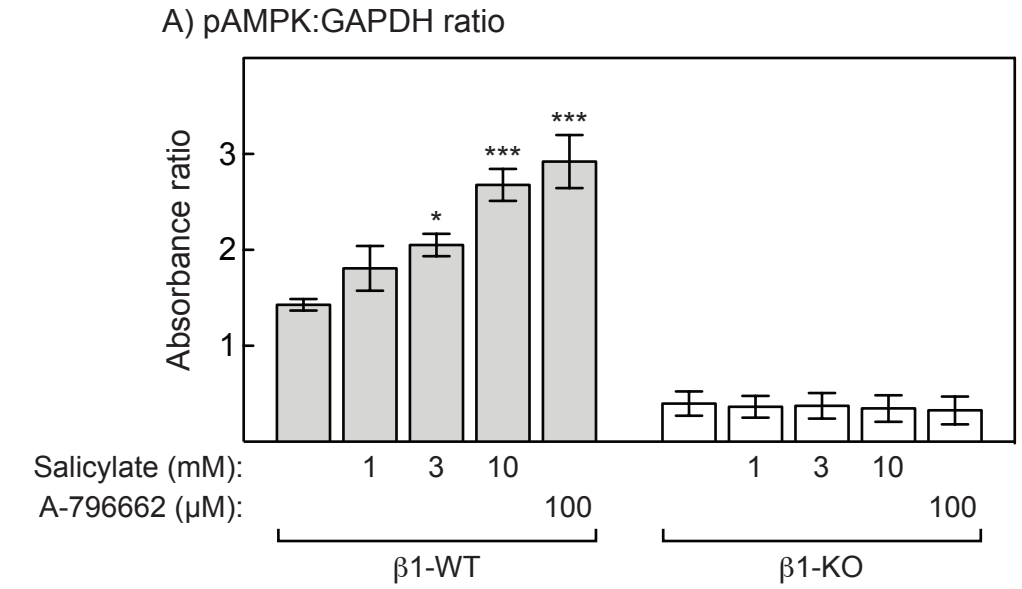
B) Effect of STO-609 on AMPK phosphorylation

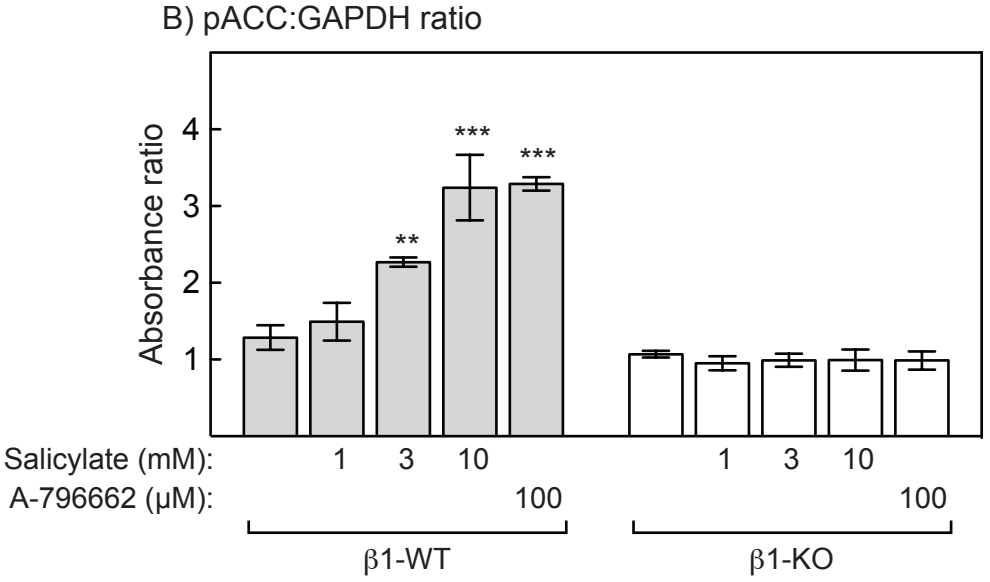


Hawley et al Figure S2



Hawley et al Figure S3





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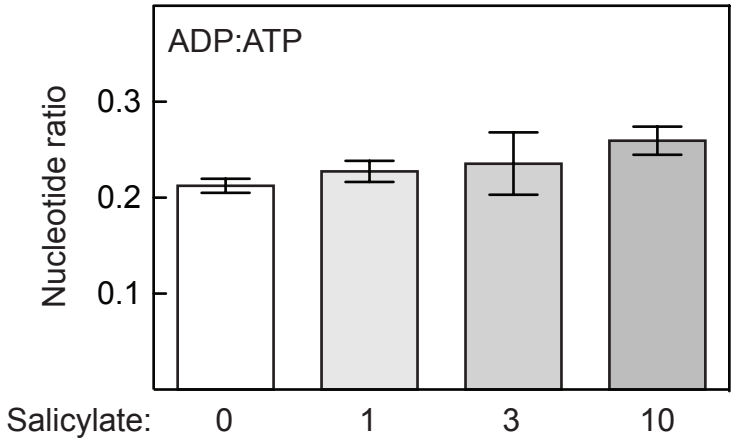
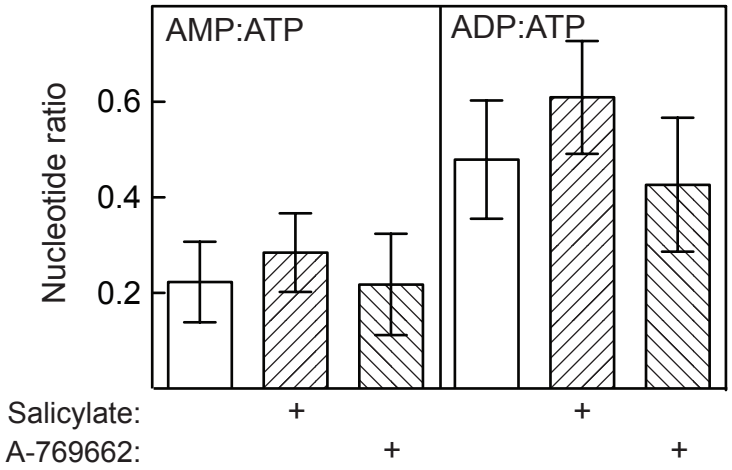
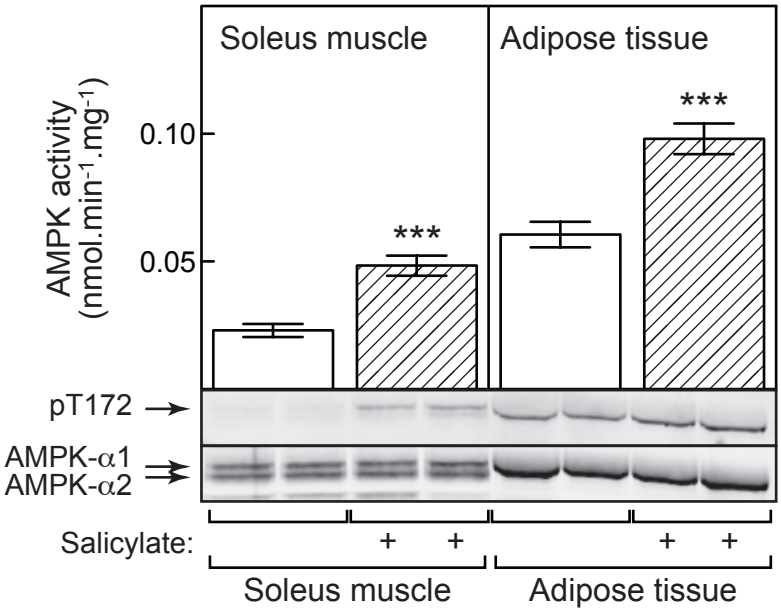
A) Isolated β1-WT hepatocytes



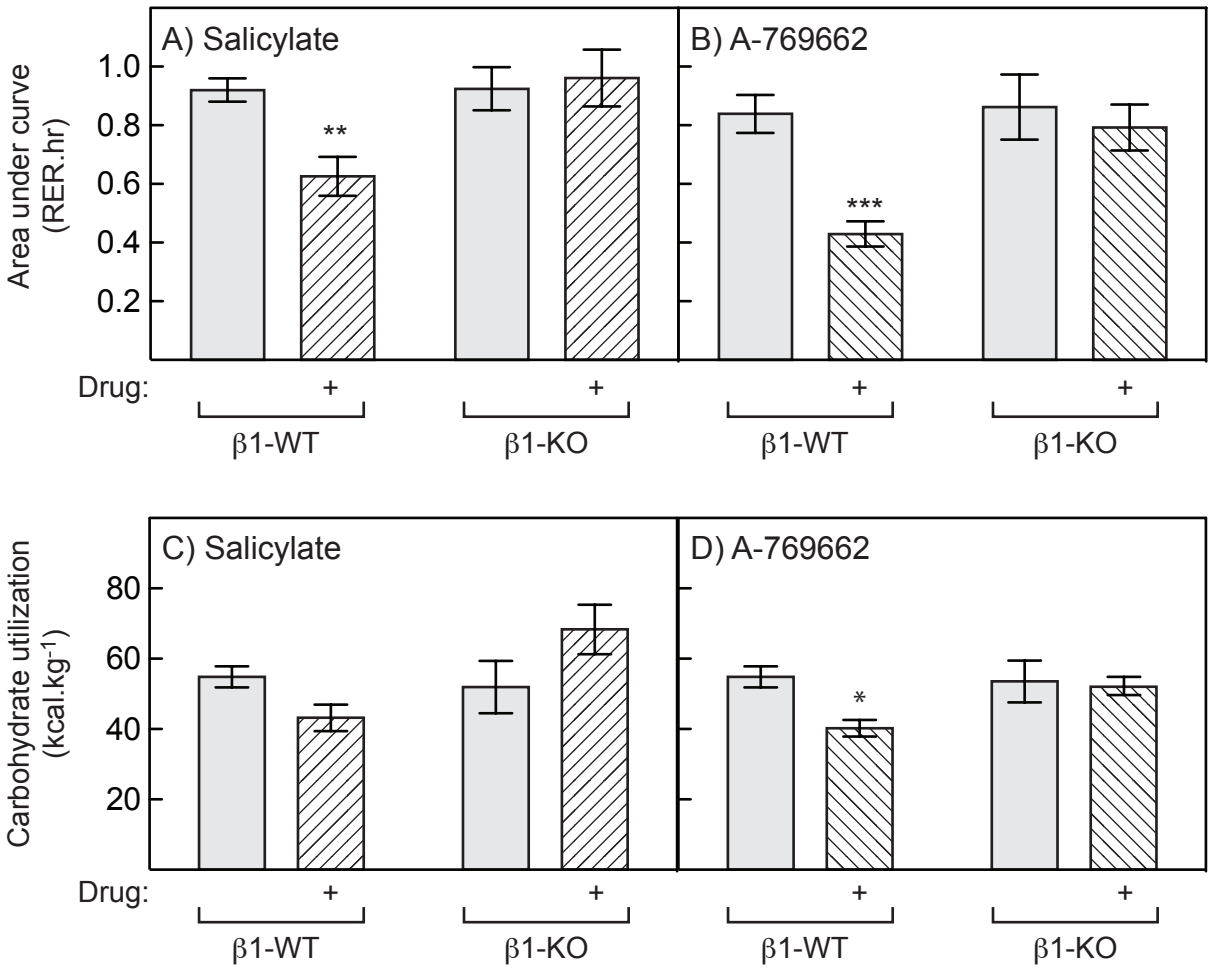
B) Liver of β1-WT mice in vivo



Hawley et al Figure S5



Hawley et al Figure S6



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